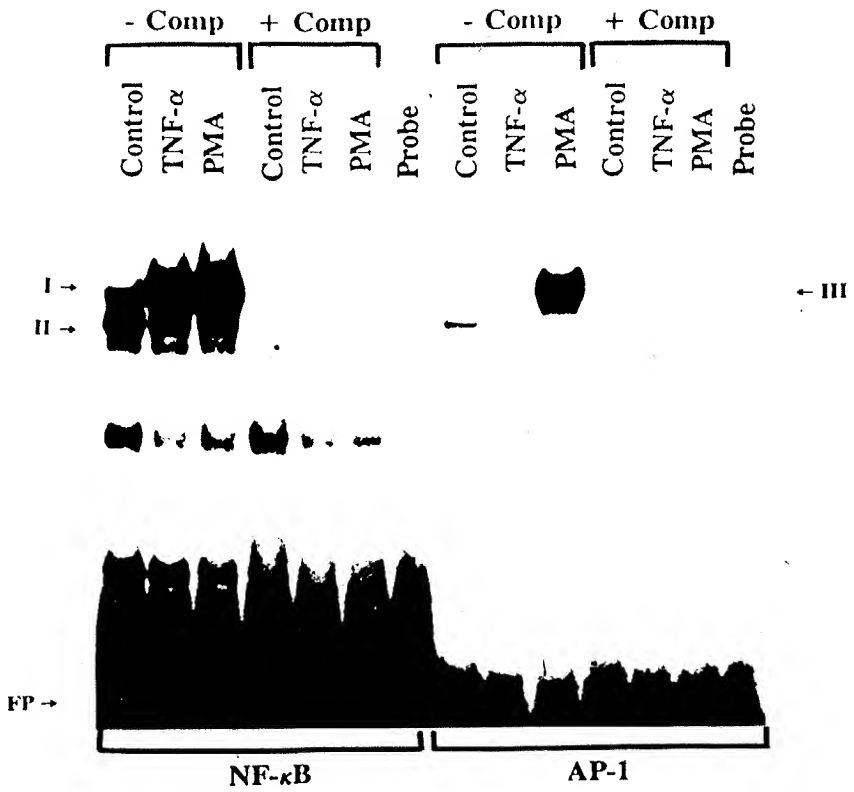

Fig. 1

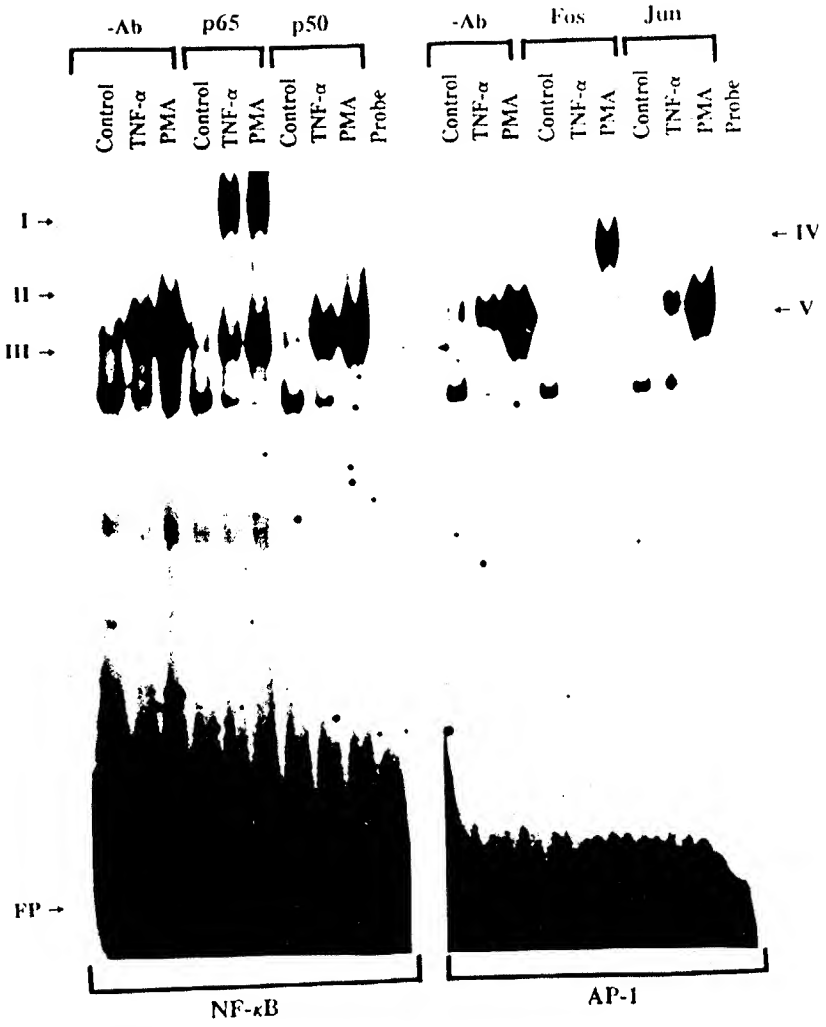

Fig. 2

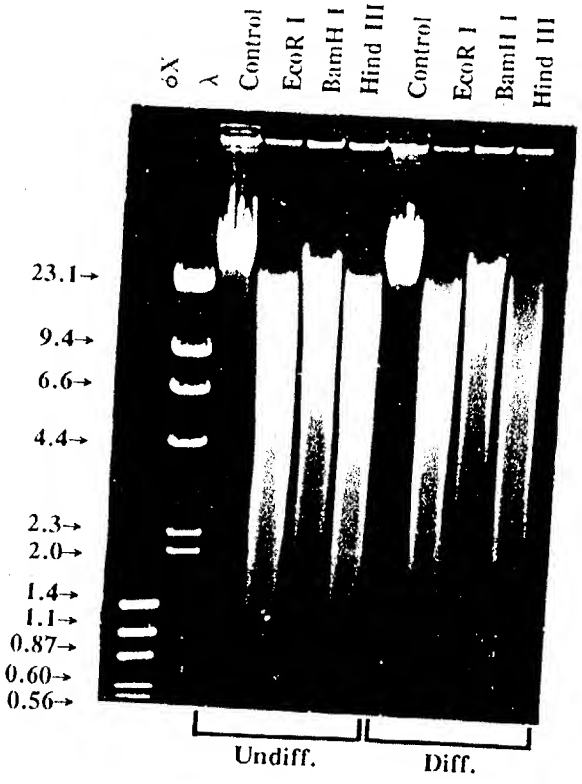

Fig. 3

1 Collecting and maintaining cells at a concentration in the range of 1-2 X 10^5/ml in culture medium,

↓

2 Treating cells with at least one test compound,

↓

3 Collecting the cell by scapping and by centrifuging at 1,000 rpm for 5 min in a table top centrifuge,

↓

4 Washing the cell pellets twice with 5 ml ice cold PBS, (phosphate buffered saline, pH 7.4) by centrifuging the cells again at 1,000 rpm,

↓

5 Resuspending the cell pellets, transferring to a first Eppendorf tube in 1 ml of ice cold PBS,

↓

Fig. 4 A

6 Centrifuging at 2,000 rpm for 5 min at 4 °C removing the PBS processing the cell pellets following according to the protein and DNA isolation steps comprising,

↓

7 Preparing Buffer A: (cell lysis buffer), [20 mM Hepes, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% NP-40, 10% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), 0.4 mM phenylmethylsulfonyl fluoride (PMSF), antipain (1 μg/ml), leupeptin (1 μg/ml)],

↓

8 Preparing Buffer B: (extraction buffer without salt), [20 mM Hepes, pH 7.9, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.4 mM PMSF, antipain (1 μg/ml), leupeptin (1 μg/ml)],

↓

9 Preparing Buffer C: (extraction buffer with salt), [20 mM Hepes, pH 7.9, 400 mM NaCl, 0.2 mM EDTA, 20% glycerol, 1 mM DTT, 0.4 mM PMSF, antipain (1 μg/ml), leupeptin (1 μg/ml)],

↓

Fig. 4 B

10 Preparing Buffer D: (cytoplasmic extraction clarification buffer), [20 mM Hepes, pH 7.9, 400 mM NaCl, 0.2 mM EDTA, 40% glycerol, 1 mM DTT, 0.4 mM PMSF, antipain (1 μg/ml), leupeptin (1 μg/ml)],

↓

11 Performing Simultaneous isolation of protein and DNA comprising the steps of,

↓

12 Resuspending the cell pellets in 100-125 μl (2 pellet vol) of Buffer A,

↓

13 Maintaining the resuspended cell pellets on ice for 10-15 min with occasional tapping,

↓

14 Pelleting the nuclei by centrifuging at 2,000 rpm for 5 min at $4^{o}C$,

↓

15 Removing the cytoplasmic supernatant fraction to a second Eppendorf tube,

↓

Fig. 4 C

16 Quick freezing on dry ice in a -86 ^{o}C freezer and store for future use,

↓

17 Washing the bottom nuclear fraction with 200-300 µl Buffer B to remove NP-40,

↓

18 Centrifuging at 2,000 rpm for 5 min at 4 ^{o}C,

↓

19 Resuspending the pelleted nuclei into 100-130 µl high salt Buffer C on ice for 45 min and mixing periodically by tapping to extract the nuclear proteins,

↓

20 Centrifuging the nuclear fraction in an Eppendorf centrifuge at 13,000 rpm for 15 min at 4 ^{o}C,

↓

21 Removing the supernatents, aliquoted, in 25 µl and quick freezing on dry ice,

↓

22 Storing at -86 ^{o}C,

↓

Fig. 4 D

23 Quick freezing the remaining pellet containing nucleic acids and other debris,

↓

24 Storing at -86 °C,

↓

25 Clarifying the cytoplasmic fraction by adding 1/3 vol of Buffer D to this fraction for 30 min at 4 °C to equilibrate the cytoplasmic proteins with NaCl,

↓

26 Centrifuging at 13,000 rpm for 15 min.,

↓

27 Removing and quick freezing the supernatants and storeing at - 86 °C,

↓

28 Performing DNA extraction and analysis comprising the steps of,

↓

29 Thawing frozen cell pellets from on ice for 10 min,

↓

30 Adding 100μl of Buffer [0.1 % SDS, 10 mM Tris-HCl, pH 7.9, 10 mM EDTA, 10 mM NaCl] for 15 min,

↓

Fig. 4 E

31 Mixing using wide bore Eppendorf pipet tips,

↓

32 Adding RNAase A for 2 h at 37 °C with gentle tapping every 30 min,

↓

33 Adding proteinase K [200 μl /ml] for 2 h at 37 °C with gentle tapping every 30 min,

↓

34 Extracting with an equal volume of TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) saturated phenol,

↓

35 Removing the upper aqueous layer was removed to another tube,

↓

36 Performing back extractions twice with 50 μl of TE,

↓

37 Collecting the DNA solutions in a fresh tube,

↓

38 Adding an equal volume of phenol/chloroform (50:50) mixture,

↓

39 Mixing by inverting repeatedly,

↓

Fig. 4 F

40 Centrifuging at 3,000 rpm for 10 min,

↓

41 Removing the upper aqueous phase,

↓

42 Extracting with an equal volume of chloroform/isoamyl (96:4) alcohol,

↓

43 Removing the upper aqueous phase containing the DNA and precipitating using 0.5 M NaCl and 3 vol of ice cold ethanol at -20 ^{o}C,

↓

44 Cenrifuging the samples at 13,000 rpm for 30 min,

↓

45 Air drying the DNA pellets,

↓

46 Dissolving DNA in 300 µl of 0.1X TE at 37 ^{o}C for 4-6 h,

↓

47 Digesting ten µl of the high molecular weight DNA from each sample for 4 h with 30 units of restriction enzymes,

↓

48 Electrophoresing on a 0.8% TAE (0.40 M Tris-acetate, 1 mM EDTA) agarose gel,

↓

Fig. 4 G

49 Staining the DNA gel with ethidium bromide,

↓

50 Photographing with UV light,

↓

51 Radiolabeling of the sequence specific oligonucleotides comprising the steps of,

↓

52 Synthesizing single stranded oligonucleotides on a DNA synthesizer and annealing with the complimentary strand by combining 4 μg of both strands in a tube with total volume of 30 μl of annealing buffer (5 mM NaCl, 10 mM Tris-HCl and 0. 2 mM EDTA),

↓

53 boiling the tubes for 5 min and slowly cooling to room temperature for 6 h,

↓

54 Heating the tubes to 55 ^{O}C for 5 min and then cooling on ice for 10 min,

↓

55 Quantitating 4 μl aliquot of the annealed oligos at 260 λ and storing the remainder at -20 ^{O}C until radiolabeled,

↓

Fig. 4 H

56 Preparing probes by radiolabeling 200 ng of annealed oligonucleotide in 15 µl of total volume containing 50 µCi of [γ-^{32}P]ATP (6,000 Ci/mmol), 20 Units of T4 polynucleotide kinase, and 1.5 µl 10X T4 polynucleotide kinase buffer for 1 h at 37 ^{o}C,

↓

57 Filling in the 5' over-hang ends with 5 Units of Klenow with 3.0 µl of 10X Klenow buffer and 0.15 mM each of dATP, dCTP, dGTP, and dTTP for 40 min at 37 ^{o}C in a reaction volume of 30 µl.

↓

58 Increasing the volume to 1 ml with sterile TE with 200 mM NaCl, pH 8.0, and the labeled oligonucleotides were purified on a NACS Prepac column, to separate the unincorporated nucleotides,

↓

59 Precipating the labeled, purified oligonucleotides overnight with 3 vol absolute ethanol at -20 ^{o}C,

↓

Fig. 4 I

60 Centrifuging at 13,000 rpm for 1 h and then vacuum drying,

↓

61 Resuspending the labeled oligonucleotide probes in 100 µl of sterile 0.1X TE buffer and storing at -20 ^{o}C,

↓

62 Electrophoretic mobility shift assay,

↓

63 Incubating 5 µg of nuclear or cytoplasmic extract, for each reaction, with 0.2-0.3 ng of [γ-^{32}P]ATP labeled oligonucleotide probe containing either NF-κB sequence (5'-gatccGGGACTTTCCGCTGGGGACTTTCCG-3') or an AP-1 consensus sequence including the PMA responsive element indicated in bold (5'-gatccG**TGACTCA**GCGCG-3'),

↓

64 Adding 3 µg of poly(dI-dC):poly(dI-dC) as a non-specific competitor and incubating with the nuclear extracts for 10 min prior to the addition of the radiolabeled probe,

↓

Fig. 4 J

65 Adding antibodies against p65, p50, c-*Fos* or c-*Jun* to the respective binding reactions for supershift assays and incubating at room temperature for 1.5 h, prior to probing with [γ-^{32}P]ATP labeled oligonucleotide for an additional 25 min at room temperature,

↓

66 Separating the bound complexes on either a 5% ,for supershift assays, or 6% ,for analytical purposes, acrylamide/bis (30:1 ratio) native gel as required and runing at 200v for 3.5 h with 0.25X TBE (0.02 M Tris-borate, 0.5 mM EDTA) as running buffer at room temperature,

↓

67 Vacuum drying the gel with heat at 80 ^{o}C and exposing them to Kodak X-OMAT film for 3-12 hours,

↓

68 Analyzing the bound and free DNA protein complexes.

Fig. 4 K